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(54) Title: METHODS FOR SELECTIVELY KILLING OR INHIBITING THE GROWTH OF CELLS EXPRESSING THE WAF1 GENE

(57) Abstract

Methods of killing or inhibiting the growth of cells are disclosed. The invention provides the administration of a WAF1 inhibitor to cells which have induced a WAF1-dependent pathway. The amount of a WAF1 inhibitor which is administered to the cells is sufficient to inhibit the growth thereof or even kill the cells. The method can include subjecting the cells to a treatment which induces a WAF1-dependent pathway.

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METHODS FOR SELECTIVELY KILLING OR INHIBITING THE GROWTH OF CELLS EXPRESSING THE WAF1 GENE

Field of the Invention

The present invention relates to the selective killing or to the selective inhibition of the growth of cells. More particularly, the present invention relates to methods and compositions useful in killing or inhibiting the growth of cells expressing the WAF1 gene. The present invention has particular utility in the field of cancer therapy.

Background of the Invention

In recent years, much progress has been made toward understanding the cell cycle of eukaryotic cells. In brief, the cell cycle consists of four phases: (1) the mitotic phase, M, in which a cell with duplicated genetic material undergoes mitosis to produce daughter cells, (2) a first gap phase, G_1, during which the cell grows and is, generally, metabolically active, (3) a synthesis phase, S, during which the cell duplicates its genetic material, and (4) a second gap phase, G_2, in which the cell prepares for mitosis and, perhaps, another cell cycle. The control or regulation of the cell cycle is a complex process involving dozens of intracellular and extracellular signals which appear to act at particular "checkpoints" at the transitions between different stages of the cell cycle. Amongst the known components of cell cycle regulation are the families of cyclins, cyclin-dependent kinases (CDKs), and cyclin-dependent kinase inhibitors (CDIs). Although the details of cell cycle control remain to be resolved, it is clear that the expression or inhibition of these molecules, as well as their stoichiometries, play a major role in controlling cell proliferation, differentiation and death (see, e.g., Hunter and Pines (1994) Cell 78:539-542).

At the same time, it has become clear that many of these same regulatory components of the normal cell cycle also play a role in oncogenesis. See, e.g., Hunter and Pines (1994) Cell 79:573-582. The p53 "tumor suppressor" gene product, for example, not only has been linked to G_1 arrest but p53 mutants have been linked to a variety of cancers (see, e.g., Vogelstein, B. and Kinzler, K.W. (1992) Cell 70:523-526; Fisher, D.E. (1994) Cell 78:539-542). This gene appears to play a role in the normal cellular response to DNA damage in mammalian cells such that, when DNA damage has occurred, the p53 gene is expressed and the cell either arrests at the G_1 stage of the cell cycle or enters the apoptotic cell death pathway. Heritable defects in p53 are

believed to be causally involved with oncogenesis either by allowing cells with damaged DNA to replicate and pass on the damaged genetic material or by preventing damaged cells from undergoing apoptosis. See, e.g., Canman, et al. (1995) Genes & Devel. 9:600-611. The p53 protein is now known to be a transcriptional regulator and several genes have been identified with putative p53-binding sites (El-Deiry et al. (1993) Cell 75:817-825).

WAF1 is a CDI which appears to be involved in the arrest of the cell cycle at a checkpoint in G_1. Because of the varying means by which it has been identified, this CDI has been known by a variety of names in the recent literature: WAF1 (Wild-type p53 activated fragment 1; El-Deiry, et al. (1993) Cell 75:817-825), Cip1 (CDK-interacting protein 1; Harper, et al. (1993) Cell 75:805-816), SDI1 (Senescent cell-derived inhibitor 1; Noda, et al. (1994) Exp. Cell Res. 211: 90-98) and p21 (21,000 Da cyclin-dependent kinase inhibitor protein; see, e.g., Jiang, et al. (1995) Oncogene 10:1855-1864). For consistency, this CDI will hereinafter be referred to as WAF1 irrespective of the nomenclature used in any references cited.

Harper et al. (1993) disclosed the nucleic acid and amino acid sequences of a human WAF1 gene and protein. These researchers found that WAF1 is a potent inhibitor of a variety of cyclin-CDK complexes and can inhibit the phosphorylation of the retinoblastoma gene product (Rb). Inhibition of the CDKs and hypophosphorylation of Rb can lead to cell cycle arrest in G_1. Consonant with this, Harper et al. found that WAF1 caused a dose-dependent *in vitro* decrease in human fibroblasts in the S phase of the cell cycle (implying WAF1-dependent G_1 arrest) and conclude that loss of WAF1 function might contribute to cell proliferation even in the presence of negative growth signals.

El-Deiry et al. (1993) also disclosed the nucleic acid and amino acid sequences of a human WAF1 gene. They noted that the gene included an upstream p53-binding site and, consonant with this, found that WAF1 was strongly induced both by p53 and by UV irradiation (which also induces p53). In addition, these authors found that WAF1 expression inhibited the growth of a variety of human tumor lines *in vitro*. These results, and others, suggested that WAF1 might be a downstream mediator of G_1 arrest controlled by the p53 tumor suppressor. Curiously, the introduction of WAF1-antisense had no effect on cell growth. Hence, WAF1 is not required for normal cell proliferation. Furthermore, in the absence of wild-type p53, induction of G_1 arrest by serum starvation or treatment with mimosine did not induce WAF1. This suggested that WAF1 expression might be one of a series of mechanisms by which cells carry out G_1 arrest. Despite these complications, these authors conclude that the "identification

of WAF1 and its regulatory region potentially provides a novel drug discovery approach: compounds that *activate* expression of WAF1 might bypass the p53 defect in tumors with endogenous p53 mutation" (emphasis added).

Sheikh et al. investigated the regulation of WAF1 expression in human breast carcinomas (Sheikh et al. (1994) Oncogene 9:3407-3415). Cells expressing wild-type p53 were found to constitutively express WAF1 at levels 26-33 fold higher than p53 mutants. In addition, exogenous mutant p53 (Val-143) counteracted the positive transcriptional effect of endogenous wild-type p53. Nonetheless, these authors found that WAF1 could be induced both by p53-independent as well as p53-dependent signaling pathways. In particular, they found that the DNA-damaging agent etoposide and serum starvation could induce both WAF1 expression and growth arrest even in p53 mutant cell lines.

Recently, Jiang et al. (1995) found that WAF1 is differentially expressed during growth, differentiation and progression in human melanoma cells. Using subtractive cDNA hybridizations and anti-p53 and anti-WAF1 antibodies, these authors found (1) lower levels of WAF1 expression in proliferating and metastatic human melanomas as compared to normal or immortalized melanocytes and (2) an increase in WAF1 protein levels in melanoma cells after induction of growth arrest and terminal differentiation (using recombinant human fibroblast β-interferon (IFN-β) and mezerein to induce terminal differentiation). In addition, levels of p53 and WAF1 were found to be inversely correlated during growth arrest and differentiation of human melanoma cells, indicating that WAF1 induction may occur independent of p53 expression. In light of these and other results, Jiang et al. suggest that agents that can *increase* WAF1 expression may prove beneficial in metastatic melanoma therapy by directly inducing an irreversible loss of proliferative capacity and terminal cell differentiation.

Using a WAF1-antisense expression vector, Nakanishi et al. investigated the effect of WAF1 inhibition on normal human fibroblasts grown to G_0 arrest *in vitro* (Nakanishi et al. (1995) Proc. Natl. Acad. Sci. (USA) 92:4352-4356). These authors found higher levels of WAF1 protein in cells arrested in G_0 than in mitogen-stimulated cells in early S phase. In addition, Nakanishi et al. found that expression of WAF1-antisense RNA caused cells arrested in G_0 to resume the cell cycle and proliferate.

The role of WAF1 in the terminal differentiation and concomitant cell cycle arrest of normal tissue was investigated by Parker et al. (1995) Science 267:1024-1027. Using *in situ* hybridization with a WAF1 probe, Parker et al. studied the tissue-specific pattern of WAF1

expression in mouse embryos at varying stages of development. The expression of WAF1 was found to correspond to the presence of post-mitotic, terminally differentiated cells (e.g., muscle, neurons). The pattern of WAF1 expression did not, however, correspond to that of p53 expression and mouse embryos lacking the p53 gene showed normal WAF1 expression and embryogenesis. Similarly, WAF1 expression in adult mice was found to be localized to terminally differentiated tissues and to be unaltered in p53-knockouts. Thus, these authors concluded that WAF1 has a role in normal terminal differentiation and embryogenesis but that WAF1 induction is not dependent upon p53 induction.

Another example of p53-independent induction of WAF1 was demonstrated in embryonic fibroblasts from p53 knock-out mice (Michieli et al. (1994) Cancer Res. 54:3391-3395). Michieli et al. found that serum, platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) and epidermal growth factor (EGF) were able to induce WAF1 in p53-deficient as well as normal cells. In contrast, γ-irradiation was able to induce WAF1 in normal cells but not in cells lacking wild-type p53. These authors conclude that WAF1 may be induced either by a p53-dependent pathway activated by DNA damage or by a p53-independent pathway activated by mitogens. The fact that WAF1 is induced by mitogens is curious in light of its apparent role in G_1 arrest and suggests that WAF1 may have an unknown function independent of G_1 arrest.

Many additional reports in the last several years have supported the hypothesis that WAF1 is an important element in regulating the cell cycle transition from G_1 to S phase and that WAF1 can be induced by a p53-dependent pathway activated by DNA damage as well as p53-independent pathways activated by cell differentiation signals. In addition, to those discussed above, p53-independent inducers of WAF1 have now been shown to include transforming growth factor β (TGFβ) in keratinocytes (Datto et al. (1995) Proc. Natl. Acad. Sci. (USA) 92:5545-5549); 12-0-tetradecanoyl phorbol-13-acetate (TPA), 1,25-dihydroxyvitamin D3 (Vit D3), retinoic acid (RA) and dimethyl-sulfoxide (DMSO) in promyelocytic leukemia cells (Jiang et al. (1994) Oncogene 9:3397-3406); and MyoD in skeletal muscle (Halevy et al. (1995) Science 267:1018-1021).

Summary of the Invention

The present invention provides methods of killing or inhibiting the growth of cells in which the WAF1 gene is being expressed. The methods comprise the administration a WAF1

inhibitor to cells in which a WAF1-dependent pathway has been induced and wherein said inhibitor is administered in an amount sufficient to kill or inhibit the growth of said cells.

In one set of embodiments, the inhibitor comprises a WAF1-antisense oligonucleotide. In particular, the oligonucleotide may be a modified oligonucleotide in which the backbone linkages, termini or bases have been modified to increase resistance to degradation or to increase binding affinity. Particularly preferred modified oligonucleotides are those containing a plurality of phosphorothioate linkages. In all embodiments in which the WAF1 inhibitor is an antisense oligonucleotide, it is preferred that the oligonucleotide is selected from the group consisting of (a) oligonucleotides comprising at least 10 consecutive bases from the WAF1 sequence disclosed as SEQ ID NO.: 1; and (b) oligonucleotides capable of hybridizing to the complements of the oligonucleotides of (a) under physiological conditions. In another set of embodiments, the inhibitor is a vector which expresses a WAF1-antisense oligonucleotide.

In an alternative set of embodiments, the inhibitor is an inhibitor of WAF1 gene transcription. In another set of embodiments, the inhibitor is an inhibitor of WAF1 protein activity such as an intracellular anti-WAF1 antibody or a fragment of either WAF1 or a CDK which competitively inhibits the formation of complexes between the WAF1 protein and endogenous CDKs. Other examples are described below.

In further embodiments, the method may include subjecting the cells to a treatment which induces a WAF1-dependent pathway. This pathway may be a p53-dependent pathway and the treatment may be one which induces p53 gene expression. Such treatments may include X-irradiation, γ-irradiation, UV-irradiation, administering to the cells an alkylating agent , administering to the cells cisplatin, administering to the cells bleomycin, doxorubicin, administering to the cells 5-fluorouracil, administering to the cells genistein, administering to the cells hydrogen peroxide, or administering to the cells methylmethane sulfonate. Alternatively, the pathway may be a p53-independent pathway. For such pathways, the treatment may include administering to the cells differentiation-inducing agents or inhibitors of DNA synthesis. Such treatments may include administering to the cells a pharmaceutical composition selected from the group consisting of PDGF, FGF, EGF, NGF, β-interferon, TGFβ, TPA, Vit D3, RA, DMSO, MyoD, IL2, rapamycin, aphidicolin, etoposide, methotrexate, cytosine arabinoside, 6-thioguanine, 6-mercaptopurine.

For all of the above-described embodiments, the method is particularly intended for use with cells which abnormally proliferate, and in a particularly important embodiment with cancer

cells in a human host. Preferred cancer cells include neuroblastoma, melanoma, epithelioma, fibroblastoma, carcinoma, leukemia and myeloma cells.

Thus, the present invention provides a method of treating a human patient having cancerous cells in which a WAF1-dependent pathway has been induced comprising administering a WAF1 inhibitor to the patient in an amount sufficient to kill or inhibit the growth of the cells. The WAF1 inhibitor may be any of those described above. In addition, the treatment may also include subjecting the patient to a treatment which induces a WAF1-dependent pathway. This additional treatment may include any of those described above. In particular, the additional treatment may include radiation or chemotherapy therapy which induces DNA damage in the cells or which induces growth arrest or differentiation of the cells. As in all embodiments described above, the preferred WAF1 inhibitor a WAF1-antisense oligonucleotide and, preferably, a modified WAF1-antisense oligonucleotide with a plurality of phosphorothioate linkages.

The invention further involves use of the foregoing compositions in the preparation of medicaments and in particular the preparation of medicaments for treating abnormal cell proliferation such as cancer.

Detailed Description of the Invention

The present invention depends, in part, upon the surprising discovery that the selective *inhibition* of the expression of WAF1 in cells in which a WAF1-dependent pathway has been induced does not lead to cell proliferation but, rather, to cell death. This result is particularly surprising in that WAF1 is believed to play a role in the G_1 arrest and/or terminal differentiation of cells and, therefore, it has previously been proposed that *inducing* the WAF1 gene or otherwise *increasing* the levels of the WAF1 protein might be a useful means of controlling the growth of cell lines *in vitro* and, more important, tumor cells *in vivo*. The present invention, in contrast, provides methods of killing or inhibiting the growth of cells by inhibiting transcription of the WAF1 gene, translation of the WAF1 mRNA transcript, or activity of the WAF1 protein. Without being bound to any particular theory of the invention, applicants believe that conditions or treatments which result in the induction of WAF1 also induce other components of a G_1 arrest or differentiation pathway. Although WAF1 is only one component in these "WAF1-dependent" pathways, it appears essential. Therefore, when WAF1 is inhibited after induction of a WAF1-

dependent pathway, it is believed that the cells cannot complete the G_1 arrest or differentiation pathways and, instead, initiate programmed cell death or apoptosis.

Clearly, the present invention is useful only with cells in which the WAF1 gene is being expressed. Thus, in some embodiments, the target cells of the invention may, without prior treatment, already be expressing WAF1. In other embodiments, however, the methods of the invention include an additional treatment which induces the expression of a WAF1-dependent pathway. Such additional treatment will typically comprise radiation or chemotherapies.

Definitions:

In order to more clearly and concisely describe the subject matter of the present invention, the following definitions are provided for specific terms used in the claims appended hereto:

WAF1. As used herein, the abbreviation "WAF1" means the human cyclin-dependent kinase inhibitor gene described in the various references cited herein and denoted as "WAF1," "Cip1," "CIP1," "SDI1," or "p21." A cDNA to one allele of WAF1 was disclosed in Harper et al. (1993) and El-Deiry et al. (1993). In addition, one WAF1 allele and the corresponding protein are disclosed herein as SEQ. ID NO.: 1 and SEQ ID NO.: 2, respectively. The translation initiation codon of this cDNA is found at base positions 76-78 and the stop codon is at positions 568-570, defining an open reading frame of 492 bases. As will be obvious to one of ordinary skill in the art, other functional alleles of WAF1 are likely to exist in the human population and are embraced by the abbreviation "WAF1" as used herein.

WAF1-dependent pathway. As used herein, the term "WAF1-dependent pathway" means a biochemical pathway in human cells in which expression of the WAF1 gene is induced. Such a pathway may require induction of WAF1 expression by the p53 tumor suppressor protein, in which case the pathway is said to be "p53-dependent." Alternatively, the pathway may not require induction of WAF1 expression by the p53 tumor suppressor protein, in which case the pathway is said to be "p53-independent."

WAF1 inhibitor. As used herein, the term "WAF1 inhibitor" means a compound which, when present in a cell, inhibits the transcription of the WAF1 gene, translation of the WAF1 mRNA transcript, or activity of the WAF1 protein product. Examples include WAF1-antisense, antibodies or fragments of antibodies which act intracellularly against the WAF1 protein or WAF1-cyclin-CDK complexes, fragments of CDKs which would act as competitive inhibitors of

WAF1 interaction with endogenous CDKs, small molecule inhibitors such as OK-1035 [Biochemical and Biophysical Research Communications 221, 207-212 (1996)] and ribozymes which inhibit WAF1 expression.

WAF1-antisense oligonucleotide. As used herein, the term "WAF1-antisense oligonucleotide" or "WAF1-antisense" describes an oligonucleotide that is an oligoribonucleotide, oligodeoxyribonucleotide or a modified oligonucleotide which hybridizes under physiological conditions to a WAF1 mRNA transcript or to WAF1 DNA and, thereby, acts as a WAF1 inhibitor. The antisense molecule, of course, is constructed and arranged so as to interfere with transcription or translation of WAF1 upon hybridization with the target. Those skilled in the art will recognize that the exact length of the antisense oligonucleotide and its degree of complementarity will depend upon the specific target selected, including the sequence of the target and the particular bases which comprise that sequence. It is preferred that the antisense oligonucleotide be selected so as to hybridize selectively with the target under physiological conditions, i.e., to hybridize substantially more with the target sequence than with any other sequence in the target cell under physiological conditions.

I. Induction of WAF1-Dependent Pathways

The p53 protein is a potent inducer of WAF1 expression. Therefore, p53 or inducers of p53 may be used to induce WAF1-dependent pathways. A variety of p53 inducers are known in the art and, because of p53's activity as a tumor suppressor, are already in use in the field of cancer therapy. Preferred p53 inducers are DNA-damaging radiation (e.g., X-rays, γ-rays, UV), DNA-damaging compounds (e.g., alkylating agents such as nitrogen mustard, chlorambucil, melphalan, cyclophosphamide, busulfan, and nitrosureas (BCNU, CCNU, methyl-CCNU); as well as etoposide, cisplatin, bleomycin, doxorubicin, 5-fluorouracil, genistein, hydrogen peroxide, and methylmethane sulfonate).

In addition, as noted above, WAF1 has been shown to be induced by p53-independent pathways. Additional preferred inducers of WAF1 therefore include WAF1-inducing growth factors and differentiation factors such as PDGF, FGF, EGF, nerve growth factor (NGF), β-interferon, TGFβ, TPA, Vit D3, RA, DMSO, MyoD and IL2, rapamycin and inhibitors of DNA synthesis such as aphidicolin, methotrexate, cytosine arabinoside, 6-thioguanine, 6-mercaptopurine.

II. Inhibition of WAF1 Expression

The present invention depends, in part, upon the discovery that the selective inhibition of the expression of WAF1 in cells in which a WAF1-dependent pathway has been induced leads to the inhibition of cell growth and/or cell death. Thus, the present invention requires that the targeted cells be subject to conflicting conditions: conditions inducing WAF1-dependent G_1 arrest or differentiation and conditions under which WAF1 expression is inhibited.

In most preferred embodiments, the WAF1-inhibiting conditions comprise treatment with WAF1-antisense oligonucleotides. Based upon SEQ. ID NO.:1, one of skill in the art can easily choose and synthesize any of a number of appropriate antisense molecules. In order to be sufficiently selective and potent for WAF1 inhibition, such WAF1-antisense oligonucleotides should comprise at least 10 bases and, more preferably, at least 15 bases. Typically, antisense molecules are between 15 and 32 bases. Most preferably, the antisense oligonucleotides comprise 18-20 bases. Although oligonucleotides may be chosen which are antisense to any region of the WAF1 gene or mRNA transcript, in preferred embodiments the antisense oligonucleotides correspond to the N-terminal or, more preferably, translation initiation region of the WAF1 mRNA or to mRNA splicing sites. In addition, WAF1 antisense may, preferably, be targeted to sites in which mRNA secondary structure is not expected (see, e.g., Sainio et al. (1994) Cell. Mol. Neurobiol. 14(5):439-457) and at which proteins are not expected to bind.

As will be obvious to one of ordinary skill in the art, the WAF1-inhibiting antisense oligonucleotides of the present invention need not be perfectly complementary to the WAF1 gene or mRNA transcript in order to be effective. Rather, some degree of mismatches will be acceptable if the antisense oligonucleotide is of sufficient length. In all cases, however, the oligonucleotides should have sufficient length and complementarity so as to selectively hybridize to a WAF1 transcript under physiological conditions. Preferably, of course, mismatches are absent or minimal. In addition, although it is not recommended, the WAF1-antisense oligonucleotides may have one or more non-complementary sequences of bases inserted into an otherwise complementary WAF1-antisense oligonucleotide sequence. Such non-complementary sequences may "loop" out of a duplex formed by a WAF1 transcript and the bases flanking the non-complementary region. Therefore, the entire oligonucleotide may retain an inhibitory effect despite an apparently low percentage of complementarity.

The WAF1-antisense oligonucleotides of the invention may be composed of deoxyribonucleotides, ribonucleotides, or any combination thereof. The 5' end of one nucleotide

and the 3' end of another nucleotide may be covalently linked, as in natural systems, via a phosphodiester internucleotide linkage. These oligonucleotides may be prepared by art recognized methods such as phosphoramidate, H-phosphonate chemistry, or methylphosphoramidate chemistry (see, e.g., Uhlmann et al. (1990) Chem. Rev. 90:543-584; Agrawal (ed.) Meth. Mol. Biol., Humana Press, Totowa, NJ (1993) Vol. 20; and U.S. Patent No. 5,149,798) which may be carried out manually or by an automated synthesizer (reviewed in Agrawal et al. (1992) Trends Biotechnol. 10:152-158).

The WAF1-antisense oligonucleotides of the invention also may include modified oligonucleotides. That is, the oligonucleotides may be modified in a number of ways which do not compromise their ability to hybridize to nucleotide sequences contained within the transcription initiation region or coding region of the WAF1 gene. The term "modified oligonucleotide" as used herein describes an oligonucleotide in which at least two of its nucleotides are covalently linked via a synthetic linkage, i.e., a linkage other than a phosphodiester linkage between the 5' end of one nucleotide and the 3' end of another nucleotide. The most preferred synthetic linkages are phosphorothioate linkages. Additional preferred synthetic linkages include alkylphosphonates, phosphorodithioates, phosphate esters, alkylphosphonothioates, phosphoramidates, carbamates, carbonates, phosphate triesters, acetamidate, and carboxymethyl esters. Oligonucleotides with these linkages or other modifications can be prepared according to known methods (see, e.g., Agrawal and Goodchild (1987) Tetrahedron Lett. 28:3539-3542; Agrawal et al. (1988) Proc. Natl. Acad. Sci. (USA) 85:7079-7083; Uhlmann et al. (1990) Chem. Rev. 90:534-583; Agrawal et al. (1992) Trends Biotechnol. 10:152-158; Agrawal (ed.) Meth. Mol. Biol., Humana Press, Totowa, NJ (1993) Vol. 20).

The term "modified oligonucleotide" also encompasses oligonucleotides with a modified base and/or sugar. For example, modified oligonucleotides include oligonucleotides having the sugars at the most 3' and/or most 5' positions attached to chemical groups other than a hydroxyl group at the 3' position and other than a phosphate group at the 5' position. Other modified ribonucleotide-containing oligonucleotides may include a 2'-O-alkylated ribose group such as a 2'-O-methylated ribose, or oligonucleotides with arabinose instead of ribose. In addition, unoxidized or partially oxidized oligonucleotides having a substitution in one nonbridging oxygen per nucleotide in the molecule are also considered to be modified oligonucleotides.

Such modifications may be at some or all of the internucleoside linkages, at either or both ends of the oligonucleotide, and/or in the interior of the molecule (reviewed in Agrawal et al. (1992) Trends Biotechnol. 10:152-158 and Agrawal (ed.) Meth. Mol. Biol., Humana Press, Totowa, NJ (1993) Vol. 20). Also considered as modified oligonucleotides are oligonucleotides having nuclease resistance-conferring bulky substituents at their 3' and/or 5' end(s) and/or various other structural modifications not found *in vivo* without human intervention. Other modifications include additions to the internucleoside phosphate linkages, such as cholesteryl or diamine compounds with varying numbers of carbon residues between the amino groups and terminal ribose.

The inhibition of WAF1 expression need not be accomplished by means of a WAF1-antisense oligonucleotide. Rather, inhibitors of WAF1 transcription or WAF1 protein activity also may be employed to the same effect. For example, antibodies or fragments of antibodies which act intracellularly against the WAF1 protein or WAF1-cyclin-CDK complexes, fragments of CDKs or recombinant genes encoding fragments of CDK which would act as competitive inhibitors of WAF1 interaction with endogenous CDKs, and ribozymes which inhibit WAF1 expression.

Without being bound to any particular theory of the invention, it is believed that treatments which result in the induction of WAF1 also induce other components of a G_1 arrest or differentiation pathway. Although WAF1 is only one component in these "WAF1-dependent" pathways, it appears essential. When WAF1 is inhibited, it is believed that the cells cannot complete the G_1 arrest or differentiation pathways and, instead, initiate programmed cell death. As a general rule, therefore, the WAF1-inhibiting conditions must be more specific to WAF1 than the WAF1-inducing conditions so that the WAF1-dependent pathway is induced while WAF1 itself is inhibited. Thus, the WAF1-inducing conditions and WAF1-inhibiting conditions should not act at the same point or level in the WAF1-dependent pathway. For example, simultaneous administration of the p53 protein (as the WAF1-inducing condition) and administration of anti-p53 antibodies (as the WAF1-inhibiting condition) would be ineffective because the conditions would largely counteract each other at the same level. In contrast, induction of the p53 gene (as the WAF1-inducing condition) and administration of WAF1 antisense oligonucleotides (as the WAF1-inhibiting condition) would be effective because p53 is a pleiotropic inducer and the WAF1-antisense oligonucleotides would not inhibit all components of the WAF1-dependent pathway.

III. Methods of Treatment in Cancer Therapy

The methods of the present invention are particularly well suited for use in the field of cancer therapy. Because current radiation and chemotherapy methods typically involve treatments which cause DNA damage and/or induce terminal differentiation of tumor cells and/or inhibit proliferation of tumor cells, these treatments, in many cases, already induce expression of the WAF1 gene. Therefore, by combining these treatments with administration of a WAF1-inhibitor, a more effective means of killing or inhibiting the growth of tumor cells is provided.

In one set of preferred embodiments, a cancer patient is treated with ionizing radiation (e.g., X-rays or γ-rays) or an agent (e.g.,doxorubicin) which causes DNA damage, induces p53 expression and, thereby, induces a p53-dependent, WAF1-dependent pathway toward cell cycle arrest. At the same time or shortly thereafter, a WAF1 inhibitor is administered to the patient. This administration may be oral, intravenous, parenteral, cutaneous or subcutaneous. The administration also may be localized to the region of the tumor by injection to or perfusion of the tumor site. Preferably, the WAF1 inhibitor is WAF1-antisense administered in a pharmaceutically acceptable carrier or a recombinant vector with a WAF1-antisense gene which expresses a WAF1-antisense oligonucleotide.

In another series of embodiments, a cancer patient is treated with compounds which induce expression of a p53-independent, WAF1-dependent pathway. These embodiments may be of particular importance because p53 mutations are associated with many cancers and mutant p53 proteins may fail to induce WAF1 expression. In preferred embodiments, the patient has been treated with an agent that induces p53-independent G_1 arrest or differentiation of the tumor cells and at the same time, or shortly thereafter, is administered a WAF1 inhibitor. Appropriate non-proliferation and differentiation agents are well known in the art and vary according to tumor type. For example, β-interferon and mezerein may be administered to melanoma patients, or TGFβ may be administered to any of a number of different types of cancer patients in order to induce a WAF1-dependent pathway. A WAF1 inhibitor, as described above, may be simultaneously or subsequently administered to kill or further inhibit the growth of the tumor cells.

The cancer may be virtually any cancer, including, but not limited to, brain cancer including glioblastoma and medulloblastoma, breast cancer, cervical cancer, colon cancer, endometrial cancer, liver cancer, lung cancer, oral cancer, prostate cancer, sarcomas, skin cancer, and renal cancer.

WAF1-antisense oligonucleotides or other WAF1 inhibitors may be administered as part of a pharmaceutical composition. Such a pharmaceutical composition may include the WAF1 inhibitor in combination with any standard physiologically and/or pharmaceutically acceptable carriers which are known in the art. The term "pharmaceutically acceptable" means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredients. The term "physiologically acceptable" refers to a non-toxic material that is compatible with a biological system such as a cell, cell culture, tissue, or organism. The characteristics of the carrier will depend on the route of administration. Physiologically and pharmaceutically acceptable carriers include diluents, fillers, salts, buffers, stabilizers, solubilizers, and other materials which are well known in the art. The pharmaceutical composition of the invention may also contain other active factors and/or agents which inhibit WAF1 expression or otherwise inhibit cell growth or increase cell death. Such additional factors and/or agents may be included in the pharmaceutical composition to produce a synergistic effect or to minimize side-effects caused by the WAF1 inhibitor of the invention.

The pharmaceutical composition of the invention may be in the form of a liposome in which WAF1-antisense oligonucleotides are combined, in addition to other pharmaceutically acceptable carriers, with amphipathic agents such as lipids which exist in aggregated form as micelles, insoluble monolayers, liquid crystals, or lamellar layers which are in aqueous solution. Suitable lipids for liposomal formulation include, without limitation, monoglycerides, diglycerides, sulfatides, lysolecithin, phospholipids, saponin, bile acids, and the like. Preparation of such liposomal formulations is within the level of skill in the art, as disclosed, for example, in U.S. Patent No. 4,235,871; U.S. Patent No. 4,501,728; U.S. Patent No. 4,837,028; and U.S. Patent No. 4,737,323.

The pharmaceutical composition of the invention may further include compounds such as cyclodextrins and the like which enhance delivery of oligonucleotides into cells, as described by Zhao et al. (in press). When the composition is not administered systemically but, rather, is injected at the site of the target cells, cationic detergents (e.g. Lipofectin) may be added to enhance uptake.

When a therapeutically effective amount of a WAF1 inhibitor is administered orally, the inhibitor will be in the form of a tablet, capsule, powder, solution or elixir. When administered in tablet form, the pharmaceutical composition of the invention may additionally contain a solid carrier such as a gelatin or an adjuvant. The tablet, capsule, and powder may contain from about

5 to 95% of a WAF1-antisense oligonucleotide and preferably from about 25 to 90% of the oligonucleotide. When administered in liquid form, a liquid carrier such as water, petroleum, oils of animal or plant origin such as peanut oil, mineral oil, soybean oil, sesame oil, or synthetic oils may be added. The liquid form of the pharmaceutical composition may further contain physiological saline solution, dextrose or other saccharide solution, or glycols such as ethylene glycol, propylene glycol or polyethylene glycol. When administered in liquid form, the pharmaceutical composition may contain from about 0.5 to 90% by weight of a WAF1-antisense oligonucleotide and preferably from about 1 to 50% of the oligonucleotide.

When a therapeutically effective amount of a WAF1 inhibitor is administered by intravenous, cutaneous or subcutaneous injection, the inhibitor will be in the form of a pyrogen-free, parenterally acceptable aqueous solution. The preparation of such parenterally acceptable solutions, having due regard to pH, isotonicity, stability, and the like, is within the skill in the art. A preferred pharmaceutical composition for intravenous, cutaneous, or subcutaneous injection should contain, in addition to the WAF1 inhibitor, an isotonic vehicle such as Sodium Chloride Injection, Ringer's Injection, Dextrose Injection, Dextrose and Sodium Chloride Injection, Lactated Ringer's Injection, or another vehicle as known in the art. The pharmaceutical composition of the present invention may also contain stabilizers, preservatives, buffers, antioxidants, or other additives known to those of skill in the art.

In preferred embodiments, when the target cells are readily accessible, administration of WAF1-antisense oligonucleotides is localized to the region of the targeted cells in order to maximize the delivery of the WAF1-antisense, minimize WAF1 inhibition in non-target cells, and minimize the amount of WAF1-antisense needed per treatment. Thus, in one preferred embodiment, administration is by direct injection at or perfusion of the site of the targeted cells, such as a tumor. Alternatively, the WAF1-antisense oligonucleotides may be adhered to small particles (e.g.,microscopic gold beads) which are impelled through the membranes of the target cells (see, e.g., U.S. Pat. No. 5,149,655).

In another series of embodiments, a recombinant gene is constructed which encodes a WAF1-antisense oligonucleotide and this gene is introduced within the targeted cells on a vector. Such a WAF1-antisense gene may, for example, consist of the normal WAF1 sequence, or a subset of the normal WAF1 sequence, operably joined in reverse orientation to a promoter region. An operable WAF1-antisense gene may be introduced on an integration vector or may be introduced on an expression vector. In order to be most effective, it is preferred that the WAF1-

antisense sequences be operably joined to a strong eukaryotic promoter which is inducible or constitutively expressed.

In all of the above-described methods of treatment, the WAF1 inhibitors are administered in therapeutically effective amounts. In addition, in those methods in which the patient also is subjected to treatment that induces a WAF1-dependent pathway, these treatments are also administered in therapeutically effective amounts. As used herein, the term "therapeutically effective amount" means the total amount of each active component of the pharmaceutical composition or method that is sufficient to show a meaningful patient benefit, i.e., the killing or inhibition of the growth of the target cells. When applied to an individual active ingredient, administered alone, the term refers to that ingredient alone. When applied to a combination, the term refers to combined amounts of the active ingredients that result in the therapeutic effect, whether administered in combination, serially or simultaneously.

The amount of WAF1 inhibitor in the pharmaceutical composition of the present invention will depend not only upon the potency of the inhibitor but also upon the nature and severity of the condition being treated, and on the nature of prior treatments which the patent has undergone. Ultimately, the attending physician will decide the amount of WAF1 inhibitor with which to treat each individual patient. Initially, the attending physician will administer low doses of the inhibitor and observe the patient's response. Larger doses of a WAF1 inhibitor may be administered until the optimal therapeutic effect is obtained for the patient, and at that point the dosage is not increased further. In preferred embodiments, the WAF1 inhibitor is a WAF1-antisense oligonucleotide and it is contemplated that the various pharmaceutical compositions used to practice the method of the present invention should contain about 1.0 μg to about 100 mg of oligonucleotide per kg body weight.

The duration of intravenous therapy using the pharmaceutical composition of the present invention will vary, depending on the severity of the disease being treated and the condition and potential idiosyncratic response of each individual patient. It is contemplated that the duration of each application of a WAF1-antisense oligonucleotide will be in the range of 12 to 24 hours of continuous intravenous administration. Ultimately the attending physician will decide on the appropriate duration of intravenous therapy using the pharmaceutical composition of the present invention.

Experimental Examples

The efficacy of the present invention was demonstrated *in vitro* on a neuroblastoma cell line using conditions known to inhibit cell proliferation and to induce differentiation as the WAF1-inducing conditions and using treatment with WAF1-antisense oligonucleotides as WAF1-inhibiting conditions. As described above, this technique did not lead to cell proliferation but, on the contrary, increased cell death. SH-SY5Y (Biedler, J. et al. (1978) Cancer Res. 38:3751-3757), a neuroblastoma cell line, was used as a model for neuronal terminal differentiation (LoPresti P. et al., (1992) Cell Growth Diff. 3:627-635; Poluha, W. et al., (1995) Oncogene 10:185-189). These cells express low levels of both the low-affinity nerve growth factor receptor (LNGFR) and the TrkA NGF receptor (Baker, D. et al., (1989) 49:4142-4146; Poluha, W. et al., (1995) Oncogene 10:185-189). SH-SY5Y cells treated with nerve growth factor (NGF) and aphidicolin, a specific and reversible inhibitor of DNA polymerases (α and δ), cease cell proliferation and extend long neurites (Jensen, L. (1987) Dev. Biol. 120:56-64; LoPresti, P. et al., (1992) Cell Growth Diff. 3:627-635). Thus, "aphidicolin + NGF" is a WAF1-inducing treatment for these cells. The differentiated cells require NGF for survival and, in the presence of NGF, are stable for 4-6 weeks. These cells express neuronal markers and resemble sympathetic neurons. In contrast, NGF alone does not stop cell proliferation and induces only slight neurite extension (Chen, J. et al., (1990) Cell Growth Diff. 1:79-85; Sonnenfeld and Ishii, (1982) J. Neurosci. Res. 8:375-391). Treatment with aphidicolin does not induce neurite extension, and the cells resume proliferation, following removal of aphidicolin.

Cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine and 100 µg/ml of gentamicin. For differentiation studies, cells were plated on Primaria (Falcon Plastics) dishes or flasks and were treated with 100 ng/ml of NGF (2.5 S; Bioproducts for Science) and/or 0.3 µM of aphidicolin. Fresh (aphidicolin+NGF)-containing medium was added every 2-3 days.

To verify the role of WAF1 in neuroblastic differentiation, expression of the mRNA for WAF1 was assessed by Northern blotting. Expression of the WAF1 mRNA transcript was up-regulated following treatment for 1 hr with aphidicolin+NGF and further increased as the aphidicolin+NGF treatment progressed. On day 6, the cells were changed from aphidicolin+NGF medium to NGF-containing medium. Despite the removal of aphidicolin from the medium, expression of WAF1 transcripts was slightly greater on day 14 than on day 6. Treatment with aphidicolin alone also induced WAF1 expression, but following removal on day

6 of aphidicolin from the medium, expression greatly declined. Treatment with NGF alone did not induce expression of WAF1. Ethidium bromide staining of RNA prior to transfer, as well as rehybridization of filters with a β-actin probe confirmed that the RNA was intact. These studies demonstrated that sustained expression of WAF1 mRNA is specifically associated with terminal differentiation of SH-SY5Y cells.

Using Western blotting, we then determined whether the level of the WAF1 protein was elevated. Treatments of SH-SY5Y cells with aphidicolin+NGF enhanced levels of WAF1 (20-fold). Expression of WAF1 protein persisted following removal of aphidicolin from the medium but at slightly lower levels. Treatment of cells with aphidicolin alone up-regulated the WAF1 (14-fold) level. Following removal of aphidicolin from the medium, expression returned to starting levels. Treatment of cells with NGF alone did not induce expression of WAF1.

As a WAF1-inhibitor, an antisense oligonucleotide with phosphorothioate linkages was employed. In addition, to enhance entry of the oligonucleotide into cells, the cationic detergent Lipofectin was used (Quattrone, A. et al., (1995) Biochemica 1:25-29; Wagner, R. (1994) Nature 372:333-335). The low concentration of detergent used in the experiments had no effect on cell viability or differentiation.

Phosphorothioate oligonucleotides (100 μM) and Lipofectin (1 mg/ml, a 1:1 (w/w) mixture of N-(1-(2,3-dioleyloxy)propyl)-N,N,N-trimethylammonium chloride and dioleoyl phosphatdylethanolamine (Gibco)) were incubated at 37°C for 15 min. The oligonucleotide-detergent mix was diluted with serum-containing medium and added to the SH-SY5Y cells. In most cases, the dilution was 1:100 giving a final oligonucleotide concentration of 1 μM. Fresh oligonucleotide-containing medium was added to the cells each day. A WAF1-antisense oligonucleotide was employed which is complementary to the region around the translational start site (5'-TCC CCA GCC GGT TCT GAC AT-3' from Oligos, Etc.). For controls, an 18-mer Control-1 (5'-TGG ATC CGA CAT GTC AGA-3') and an antisense oligonucleotide directed against *M. tuberculosis* (5'-CGC TTC ATC CTG CCG TGT CGG-3'), Control-2, were employed. Expression in the of WAF1-induced cells was reduced by the antisense oligonucleotide but not by two control oligonucleotides. As judged by Western blotting, the antisense oligonucleotide, but not the control, decreased WAF1 expression by 2.0-2.5 fold.

The antisense oligonucleotide had no apparent effect on the morphology or proliferation of control cells. However, cells treated with both WAF1-inducing and WAF1-inhibiting conditions differentiated into neuronal cells but the number of live cells per dish was much less

than for cells treated with only WAF1-inducing conditions. Cells were stained with Hoechst 33342 to assay for apoptotic bodies which are characteristic of programmed cell death (Gregory, C. et al., (1991) Nature 349:612-614). In four independent experiments, the percentage of cells treated with both WAF1-inducing and WAF1-inhibiting conditions which exhibited apoptotic bodies was roughly twice that for cells treated with only inducing conditions or for cells treated with the inducing conditions and control oligonucleotides.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

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(F) ZIP: 01545

(ii) TITLE OF INVENTION: METHODS AND PRODUCTS FOR SELECTIVELY KILLING OR INHIBITING THE GROWTH OF CELLS EXPRESSING THE WAF1 GENE

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:

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(E) COUNTRY: USA

(F) ZIP: 02210

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 60/001,248

(B) FILING DATE: 20-JULY-1995

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: GATES, EDWARD R.

(B) REGISTRATION NUMBER: 31,616

(C) REFERENCE/DOCKET NUMBER: W0461/7028WO

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 617-720-3500

(B) TELEFAX: 617-720-2441

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2121 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: HOMO SAPIENS

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 76..570

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GCCGAAGTCA GTTCCTTGTG GAGCCGGAGC TGGGCGCGGA TTCGCCGAGG CACCGAGGCA 60

CTCAGAGGAG GCGCC ATG TCA GAA CCG GCT GGG GAT GTC CGT CAG AAC CCA 111
Met Ser Glu Pro Ala Gly Asp Val Arg Gln Asn Pro
1 5 10

TGC GGC AGC AAG GCC TGC CGC CGC CTC TTC GGC CCA GTG GAC AGC GAG 159
Cys Gly Ser Lys Ala Cys Arg Arg Leu Phe Gly Pro Val Asp Ser Glu
15 20 25

CAG CTG AGC CGC GAC TGT GAT GCG CTA ATG GCG GGC TGC ATC CAG GAG 207
Gln Leu Ser Arg Asp Cys Asp Ala Leu Met Ala Gly Cys Ile Gln Glu
30 35 40

GCC CGT GAG CGA TGG AAC TTC GAC TTT GTC ACC GAG ACA CCA CTG GAG 255
Ala Arg Glu Arg Trp Asn Phe Asp Phe Val Thr Glu Thr Pro Leu Glu
45 50 55 60

GGT GAC TTC GCC TGG GAG CGT GTG CGG GGC CTT GGC CTG CCC AAG CTC 303
Gly Asp Phe Ala Trp Glu Arg Val Arg Gly Leu Gly Leu Pro Lys Leu
65 70 75

TAC CTT CCC ACG GGG CCC CGG CGA GGC CGG GAT GAG TTG GGA GGA GGC 351
Tyr Leu Pro Thr Gly Pro Arg Arg Gly Arg Asp Glu Leu Gly Gly Gly
80 85 90

AGG CGG CCT GGC ACC TCA CCT GCT CTG CTG CAG GGG ACA GCA GAG GAA 399
Arg Arg Pro Gly Thr Ser Pro Ala Leu Leu Gln Gly Thr Ala Glu Glu
95 100 105

GAC CAT GTG GAC CTG TCA CTG TCT TGT ACC CTT GTG CCT CGC TCA GGG 447
Asp His Val Asp Leu Ser Leu Ser Cys Thr Leu Val Pro Arg Ser Gly
110 115 120

GAG CAG GCT GAA GGG TCC CCA GGT GGA CCT GGA GAC TCT CAG GGT CGA 495
Glu Gln Ala Glu Gly Ser Pro Gly Gly Pro Gly Asp Ser Gln Gly Arg
125 130 135 140

AAA CGG CGG CAG ACC AGC ATG ACA GAT TTC TAC CAC TCC AAA CGC CGG 543
Lys Arg Arg Gln Thr Ser Met Thr Asp Phe Tyr His Ser Lys Arg Arg
145 150 155

CTG ATC TTC TCC AAG AGG AAG CCC TAATCCGCCC ACAGGAAGCC TGCAGTCCTG 597
Leu Ile Phe Ser Lys Arg Lys Pro
160 165

GAAGCGCGAG GGCCTCAAAG GCCCGCTCTA CATCTTCTGC CTTAGTCTCA GTTTGTGTGT 657

CTTAATTATT ATTTGTGTTT TAATTTAAAC ACCTCCTCAT GTACATACCC TGGCCGCCCC 717

CTGCCCCCCA GCCTCTGGCA TTAGAATTAT TTAAACAAAA ACTAGGCGGT TGAATGAGAG 777

GTTCCTAAGA GTGCTGGGCA TTTTTATTTT ATGAAATACT ATTTAAAGCC TCCTCATCCC 837

GTGTTCTCCT TTTCCTCTCT CCCGGAGGTT GGGTGGGCCG GCTTCATGCC AGCTACTTCC 897

TCCTCCCCAC TTGTCCGCTG GGTGGTACCC TCTGGAGGGG TGTGGCTCCT TCCCATCGCT 957

GTCACAGGCG GTTATGAAAT TCACCCCCTT TCCTGGACAC TCAGACCTGA ATTCTTTTTC 1017

ATTTGAGAAG TAAACAGATG GCACTTTGAA GGGGCCTCAC CGAGTGGGGG CATCATCAAA 1077

AACTTTGGAG TCCCCTCACC TCCTCTAAGG TTGGGCAGGG TGACCCTGAA GTGAGCACAG 1137

CCTAGGGCTG AGCTGGGGAC CTGGTACCCT CCTGGCTCTT GATACCCCCC TCTGTCTTGT 1197

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TGACCTGCAC TGGGGAGCCC GTCTCAGTGT TGAGCCTTTT CCCTCTTTGG CTCCCCTGTA 1317

CCTTTTGAGG AGCCCCAGCT TACCCTTCTT CTCCAGCTGG GCTCTGCAAT TCCCCTCTGC 1377

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GAAGGGCACC CTAGTTCTAC CTCAGGCAGC TCAAGCAGCG ACCGCCCCCT CCTCTAGCTG 1557

TGGGGGTGAG GGTCCCATGT GGTGGCACAG GCCCCCTTGA GTGGGGTTAT CTCTGTGTTA 1617

GGGGTATATG ATGGGGGAGT AGATCTTTCT AGGAGGGAGA CACTGGCCCC TCAAATCGTC 1677

CAGCGACCTT CCTCATCCAC CCCATCCCTC CCCAGTTCAT TGCACTTTGA TTAGCAGCGG 1737

AACAAGGAGT CAGACATTTT AAGATGGTGG CAGTAGAGGC TATGGACAGG GCATGCCACG 1797

TGGGCTCATA TGGGGCTGGG AGTAGTTGTC TTTCCTGGCA CTAACGTTGA GCCCCTGGAG 1857

GCACTGAAGT GCTTAGTGTA CTTGGAGTAT TGGGGTCTGA CCCCAAACAC CTTCCAGCTC 1917

CTGTAACATA CTGGCCTGGA CTGTTTTCTC TCGGCTCCCC ATGTGTCCTG GTTCCCGTTT 1977

CTCCACCTAG ACTGTAAACC TCTCGAGGGC AGGGACCACA CCCTGTACTG TTCTGTGTCT 2037

TTCACAGCTC CTCCCACAAT GCTGAATATA CAGCAGGTGC TCAATAAATG ATTCTTAGTG 2097

ACTTTAAAAA AAAAAAAAAA AAAA 2121

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 164 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Ser Glu Pro Ala Gly Asp Val Arg Gln Asn Pro Cys Gly Ser Lys
1 5 10 15

Ala Cys Arg Arg Leu Phe Gly Pro Val Asp Ser Glu Gln Leu Ser Arg
20 25 30

Asp Cys Asp Ala Leu Met Ala Gly Cys Ile Gln Glu Ala Arg Glu Arg

35 40 45

Trp Asn Phe Asp Phe Val Thr Glu Thr Pro Leu Glu Gly Asp Phe Ala
50 55 60

Trp Glu Arg Val Arg Gly Leu Gly Leu Pro Lys Leu Tyr Leu Pro Thr
65 70 75 80

Gly Pro Arg Arg Gly Arg Asp Glu Leu Gly Gly Gly Arg Arg Pro Gly
85 90 95

Thr Ser Pro Ala Leu Leu Gln Gly Thr Ala Glu Glu Asp His Val Asp
100 105 110

Leu Ser Leu Ser Cys Thr Leu Val Pro Arg Ser Gly Glu Gln Ala Glu
115 120 125

Gly Ser Pro Gly Gly Pro Gly Asp Ser Gln Gly Arg Lys Arg Arg Gln
130 135 140

Thr Ser Met Thr Asp Phe Tyr His Ser Lys Arg Arg Leu Ile Phe Ser
145 150 155 160

Lys Arg Lys Pro

CLAIMS

1. A method of killing or inhibiting the growth of cells comprising administering a WAF1 inhibitor to cells in which a WAF1-dependent pathway has been induced and wherein said inhibitor is administered in an amount sufficient to kill or inhibit the growth of said cells.

2. A method as in claim 1 wherein said inhibitor comprises a WAF1-antisense oligonucleotide.

3. A method as in claim 2 wherein said inhibitor is a modified oligonucleotide containing a plurality of phosphorothioate linkages.

4. A method as in either claim 2 or claim 3 wherein said oligonucleotide is selected from the group consisting of

(a) oligonucleotides comprising at least 10 consecutive bases from SEQ ID NO.: 1; and

(b) oligonucleotides capable of hybridizing to the complements of the oligonucleotides of (a) under physiological conditions.

5. A method as in claim 1 wherein said inhibitor is a vector which expresses a WAF1-antisense oligonucleotide.

6. A method as in claim 1 wherein said inhibitor is an inhibitor of WAF1 gene transcription.

7. A method as in claim 1 wherein said inhibitor is an inhibitor of WAF1 protein activity.

8. A method as in claim 1 wherein said method further comprises subjecting said cells to a treatment which induces a WAF1-dependent pathway.

9. A method as in claim 8 wherein said WAF1-dependent pathway is a p53-dependent pathway and said treatment induces p53 gene expression.

10. A method as in claim 9 wherein said treatment is selected from the group consisting of X-irradiation, γ-irradiation, UV-irradiation, administering to said cells an alkylating agent , administering to said cells cisplatin, administering to said cells bleomycin, doxorubicin, administering to said cells 5-fluorouracil, administering to said cells genistein, administering to said cells hydrogen peroxide, and administering to said cells methylmethane sulfonate.

11. A method as in claim 8 wherein said WAF1-dependent pathway is a p53-independent pathway.

12. A method as in claim 11 wherein said treatment comprises administering to said cells differentiation-inducing agents.

13. A method as in claim 11 wherein said treatment comprises administering to said cells inhibitors of DNA synthesis.

14. A method as in claim 11 wherein said treatment comprises administering to said cells a pharmaceutical composition selected from the group consisting of PDGF, FGF, EGF, NGF, β-interferon, TGFβ, TPA, Vit D3, RA, DMSO, MyoD, IL2, rapamycin, aphidicolin, etoposide, methotrexate, cytosine arabinoside, 6-thioguanine, 6-mercaptopurine.

15. A method as in claim 1 wherein said cells are cancer cells in a human host.

16. A method as in claim 15 wherein said cancer cells are selected from the group consisting of neuroblastoma, melanoma, epithelioma, fibroblastoma, carcinoma, leukenia and myeloma cells.

17. A method of treating a human patient having cancerous cells in which a WAF1-dependent pathway has been induced comprising

administering a WAF1 inhibitor to said patient in an amount sufficient to kill or inhibit the growth of said cells.

18. A method as in claim 17 further comprising

subjecting said patient to a treatment which induces a WAF1-dependent pathway.

19. A method as in claim 18 wherein said treatment comprises radiation therapy which induces DNA damage in said cells.

20. A method as in claim 19 wherein said treatment comprises chemotherapy which induces growth arrest or differentiation of said cells.

21. A method as in any one of claims 17 to 20 wherein said inhibitor is a WAF1-antisense oligonucleotide.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/11886

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 31/785, 38/00, 38/18; C12N 13/00
US CL :514/2,44; 435/172.1, 173.1, 173.7, 240.2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/2,44; 435/172.1, 173.1, 173.7, 240.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN
search terms: waf1, cell death, oligonucleotide, p21, Cip1, CDI1

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	MANFREDI, et al. The Cyclin-Dependent Kinase Inhibitor p21 as a Target for Differentiation Therapy. Molecular and Cellular Differentiation. 1996, Vol. 4, No. 1, pages 33-45, especially pages 35-36.	1-21
X	US 5,302,706 A (SMITH) 12 April 1994, Figures 1, 3, 5.	2-5, 21

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 30 AUGUST 1996	Date of mailing of the international search report 09 OCT 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer MATTHEW LATIMER Telephone No. (703) 308-0196

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